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Experimental Hematology

The Concentration and Resolution of Primitive Hemopoietic Cells from Normal Mouse Bone Marrow by Negative Selection Using Monoclonal Antibodies and Dynabead Monodisperse Magnetic Microspheres

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Abstract. High proliferative potential colony-forming cells (HPP-CFC) detected in clonal agar culture in the presence of the combined stimulus of colony-stimulating factor 1 (CSF-1) + interleukin 3 (IL-3) + interleukin 1_α (IL-1_α) are closely related to developmentally early progenitor cells capable of reconstituting the hemopoietic system of lethally irradiated mice following transplantation. Flow cytometric analysis and sorting of normal, unperturbed bone marrow has shown that HPP-CFC are B220$^-$ and 7/4$^-$, whereas the committed progenitors of the macrophage lineage responsive to CSF-1 alone (CFC_{CSF-1}) are B220$^-$ and 7/4$^+$. Negative immunomagnetic selection using an anti-7/4, anti-B220 antibody cocktail and second-antibody-coupled Dynabead microspheres to replace flow cytometry results in the highly reproducible and specific enrichment of HPP-CFC, and simultaneous resolution of HPP-CFC from CFC_{CSF-1}. The tenfold enrichment of HPP-CFC compared with unfractionated bone marrow cell suspensions was comparable to that obtained by fluorescence-activated cell sorting. Enrichment was achieved with negligible loss of HPP-CFC at the immunomagnetic bead selection step, and 65% of HPP-CFC were recovered. The method is rapid, highly reproducible, and efficient, and has wide application to the separation of rare hemopoietic cells from normal bone marrow.

Key words: Hemopoietic stem cells — Immunomagnetic beads — Cell separation — HPP-CFC

High proliferative potential colony-forming cells (HPP-CFC), which are detected in clonal agar culture by their ability to form colonies in the presence of the combined stimulus of the macrophage lineage-specific growth factor, colony-stimulating factor 1 (CSF-1), and other hemopoietic growth factors, which synergize with CSF-1 [1–3], are present in normal bone marrow at very low frequencies (<0.1%), and share properties associated with a cohort of multipotential transplantable hemopoietic cells with extensive hemopoietic reconstituting capacity [4].

Fluorescence-activated cell sorting of normal mouse bone marrow on the basis of the expression of the Qa-m7 antigenic determinant, for example, resulted in significant enrichment of HPP-CFC, and cloning efficiencies up to 5% HPP-CFC were achieved [5]. By exploiting the relative resistance of HPP-CFC to the cytotoxic effects of 5-fluorouracil (5-FU), which results in a substantial expansion of the HPP-CFC population in regenerating marrow with an accompanying increase in the level of Qa-m7 surface antigen expression, preparations containing >20% HPP-CFC can be obtained [4], but recovery is low.

In common with others attempting to prepare highly purified populations of rare hemopoietic progenitors for further study, we have found that flow cytometric cell sorting is a very powerful analytical tool, but is limited in its preparative applications; in order to achieve high purity, target cell recovery has to be severely compromised, and the time required to sort substantial numbers of highly purified cells is prohibitive.

Recently, monodisperse magnetic polymer particles specifically intended for use in cell separative procedures have been developed and have become commercially available. Numerous reports have appeared in the literature confirming their efficacy in purging tumor cells [6–8] and lymphocytes [9] from bone marrow, and in isolating lymphocyte subsets from peripheral blood [10, 11] by both positive and negative immunomagnetic selection. The very low levels of nonspecific binding of these beads suggested that in conjunction with appropriate antibodies, immunomagnetic selection could be a useful preenrichment step, and in some cases an alternative, to fluorescence-activated cell sorting for the purification and enrichment of rare primitive hemopoietic progenitor cells such as HPP-CFC.

Using antibody-conjugated Dynabeads (Dynal AS, Norway) and selected monoclonal antibodies, we have been able to resolve HPP-CFC from colony-forming cells of low proliferative potential responsive to the macrophage lineage-specific growth factor CSF-1 alone. The enrichments of HPP-CFC from normal bone marrow are comparable to those obtained by fluorescence-activated cell sorting but the recovery of target cells is significantly higher.

Materials and methods

Preparation of murine bone marrow cell suspensions. Three- to 4-month-old (C57BL/6J × DBA2) F_1 mice, bred and maintained under specific pathogen-free conditions at the Peter MacCallum Cancer Institute, were anesthetized (Fluothane; ICI Australia Operations

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Proprietary, Limited) and killed by cervical dislocation. Marrow cells were flushed from the femoral shafts with cold HEPES-buffered balanced salt solution (BSS) containing 2% newborn calf serum by using a 1-ml syringe fitted with a 23-gauge needle. Groups of at least three mice were used per experiment.

Monoclonal antibodies. The rat monoclonal antibody directed against the 7/4 murine differentiation antigen [12] was obtained from Dr. S. Gordon, Sir William Dunn School of Pathology, University of Oxford, England; the RA3.6B2 rat monoclonal antibody directed against the B220 antigen [13] was obtained from Dr. W. Langdon, Walter and Eliza Hall Institute, Melbourne. These antibodies were obtained as tissue culture supernatants, and were used at previously determined optimal concentrations after dilution in phosphate-buffered saline (PBS; pH 7.4, 290 mOsmol) supplemented with 5% newborn bovine serum (binding buffer).

Unconjugated or fluoresceinated affinity-purified mouse serum adsorbed F(ab')$_2$ goat anti-rat IgG was purchased from Kirkegaard and Perry Laboratories Incorporated, Gaithersburg, Maryland.

Flow cytometric analysis and cell sorting. Low-density bone marrow cells (<1.085 g/cm^3) were isolated by discontinuous density gradient centrifugation using Nycodenz (Nyegaard, Oslo, Norway) exactly as previously described [4]. These cells were washed and resuspended at 5×10^7 cells/ml by adding equal volumes of binding buffer and anti-7/4 or anti-B220 antibodies. Simultaneous labeling with both antibodies was performed by incubating the cells in equal volumes of each antibody preparation. After incubation for 30 min on ice, the labeled cells were washed three times in binding buffer, resuspended, and incubated with fluoresceinated second antibody for a further 30 min on ice. The cells were then washed a further three times, resuspended at 10^6/ml in binding buffer supplemented with 0.25% newborn bovine serum, and held on ice for flow cytometric analysis and cell sorting.

Cells with the predetermined light scattering properties of hemopoietic progenitors [5] were analyzed and sorted at 4°C at a rate of 2000 cells/s using an Ortho System 50H Cytofluorograph with a 2150 computer system (Ortho Diagnostic Systems, Westwood, Massachusetts). Low angle forward light scatter and 90° light scatter were measured using a helium–neon laser. Green fluorescence was measured using a Spectra Physics series 2000 argon ion laser tuned to 488 nm at a power of 400 mW, and a fluorescein isothiocyanate (FITC) filter arrangement with peak transmittance at 530 nm and a bandwidth of 10 nm.

Preparation of immunomagnetic beads. Goat anti-rat F(ab')$_2$ IgG was covalently bound to uncoated monodisperse magnetic polystyrene beads (M-450 Dynabeads) exactly according to the manufacturer's instructions.

Uncoated beads were washed in acetone and were activated by incubation for 20 h at room temperature with end-over-end rotation in the presence of 0.75 mmol pyridine and 0.3 mmol *p*-toluenesulfonyl chloride per ml of undiluted bead suspension (4×10^8 beads/ml) in acetone. The beads were collected using a Dynal magnetic particle concentrator (MPC-1), resuspended, washed three times in acetone, transferred back to water, and resuspended in 10 ml of 1 mM HCl.

Covalent coupling of the antibody was performed by washing and resuspending the stored, activated beads in sterile distilled water and then adding an equal volume of antibody dissolved in 0.2 M borate buffer (pH 9.5) at a concentration of 150 μg protein/ml to achieve an antibody : bead ratio of 75 μg protein per 15 mg beads. Following incubation of this suspension for 22 h of end-over-end rotation at room temperature, the conjugated beads were sequentially washed with 5-ml aliquots of 0.1 M PBS (10 min); 1 M ethanolamine-HCl, pH 9.5, containing 0.1% Tween 20 (2 h); 0.05 M Tris in 0.1 M NaCl, pH 7.5, with added 0.1% bovine serum albumin (BSA) and 0.1% Tween 20 (12 h); and 0.05 M Tris in 0.1 M NaCl, pH 7.5 plus 0.1% BSA (2 h). The conjugated beads were then washed, resuspended at 2×10^8 beads/ml in PBS supplemented with 0.25% newborn calf serum, and stored at 4°C for use. Beads prepared in this way have been stored for >3 months without showing any loss of activity or specificity.

Negative immunomagnetic selection. Unless otherwise specified, negative immunomagnetic selection was performed by incubating primary-antibody labeled cells with anti-rat IgG-coupled beads at 4°C for 45 min of end-over-end rotation at a predetermined bead: cell ratio. The test tube containing the bead-conjugated cell suspension was then placed in a Dynal MPC-1 magnetic particle concentrator for 2 min. Bead-rosetted cells were attracted to the wall of the tube, and the unrosetted cells remaining in suspension were harvested using a Pasteur pipette. Cell counts and recovery were determined prior to plating.

Growth factors and clonal agar assay. The macrophage lineage-specific growth factor, CSF-1 (sp. act. approximately 8×10^7 U/mg protein) was purified from pregnant mouse uterus extract prepared in this laboratory [14] using anti-CSF-1 immunoabsorbent column chromatography [15] by E.R. Stanley (Albert Einstein College of Medicine, New York). Purified recombinant murine interleukin 3 (IL-3; sp. act. 2.3×10^6 U/μg protein) [16] was a gift of Drs. A. Hapel and I. Young (Australian National University, Canberra), and purified human recombinant interleukin 1$_\alpha$ (IL-1$_\alpha$; 10^9 conversion assay U/μg protein) was supplied by Immunex Corporation (Seattle, Washington). All factors were used at predetermined optimal concentrations. For a 1.5-ml culture volume the amounts of factor were: CSF-1, 10^3 U; IL-3, 25 U; and IL-1$_\alpha$, 2×10^6 U.

Fractionated and unfractionated bone marrow cells were assayed for CFC$_{CSF\text{-}1}$ and HPP-CFC$_{CSF\text{-}1+IL\text{-}3+IL\text{-}1\alpha}$ in a double-layer nutrient agar culture system using 35-mm plastic petri dishes (Bunzl, Camden Park, South Australia). The medium used in this study was the alpha modification of Eagle's minimal essential medium supplemented with vitamins (×2), L-glutamine, and 20% newborn calf serum. Growth factors were included in a 1-ml underlay with a final agar (bacto-agar; Difco, Detroit, Michigan) concentration of 0.5%. Target cells were included in a 0.5-ml overlay with a final agar concentration of 0.33%. Culture dishes were incubated for 12–14 days in a gas phase of 7% O_2, 10% CO_2, and 83% N_2 exactly as previously described [17] for optimal colony formation.

Target cells were defined according to their responsiveness to growth factors. CFC$_{CSF\text{-}1}$ are low proliferative potential colony-forming cells (LPP-CFC) typically forming colonies of <0.25 mm in diameter, containing approximately 50–5000 cells per colony. HPP-CFC$_{CSF\text{-}1+IL\text{-}3+IL\text{-}1\alpha}$ typically formed colonies of >0.5 mm in diameter, containing 50,000 cells or more per colony.

Results

Of the many monoclonal antibodies known to react with determinants present on murine bone marrow cells, two were selected that exhibited specificities conducive to negative immunoselection of primitive hemopoietic cells. These were the rat anti-mouse monoclonal antibodies directed against 7/4 antigen [12] and B220 antigen [13].

7/4 antigen is expressed on approximately 60% of murine marrow cells and is restricted to the relatively mature cells of the neutrophil/macrophage lineage [12]. Although data were not available on the distribution of this marker on hemopoietic progenitor cells, the available evidence suggested that primitive multipotential cells would be 7/4$^-$.

The B220 antigenic determinant has been characterized more precisely. It is expressed only on cells of the B-lymphocyte lineage from the level of the pre-B cell to mature B-lymphocytes. Up to 40% of bone marrow cells are B220$^+$, but transplantable multipotential hemopoietic cells including spleen colony-forming units (CFU-S) are B220$^-$ [13, 18].

Flow cytometric analysis and sorting of low-density bone marrow cells of pre-determined light scattering characteristics demonstrated that 7/4 antigen (Table 1) and B220 antigen (Table 2) were not expressed on HPP-CFC. CFC$_{CSF\text{-}1}$ were also found to be B220$^-$ (Table 2); however, the majority

Table 1. Enrichment of normal bone marrow colony-forming cells by discontinuous density gradient centrifugation and cell sorting on the basis of light scatter and 7/4 surface antigen expression

		Nucleated cell recovery (percent)	Colonies per 2500 cells[a]	
			CFC_{CSF-1}	HPP-CFC
Unfractionated bone marrow		100	17.3 ± 3.0	3.7 ± 0.9
Low-density cells		37.6	56.7 ± 2.2 (123.3%)[b]	10.0 ± 3.8 (101.6%)
Low-density cells selected by light scatter and fluorescence				
Region 1	$7/4^-$	0.5	66.7 ± 17.6 (1.9%)	86.7 ± 8.8 (11.7%)
Region 2	$7/4^-$	0.5	43.3 ± 14.5 (1.3%)	53.3 ± 14.5 (7.2%)
Region 3	$7/4^-$	0.5	53.3 ± 8.8 (1.5%)	33.3 ± 3.3 (4.5%)
Region 4	$7/4^{\pm}$	0.4	200.0 ± 38.2 (4.6%)	26.7 ± 7.3 (2.9%)
Region 5	$7/4^+$	0.9	116.0 ± 16.5 (6.0%)	0.6 (0.1%)
Region 6	$7/4^+$	1.6	76.7 ± 12.5 (7.1%)	0

[a] Means ± standard error of the mean (SEM) from a single experiment plated in triplicate.
[b] Progenitor cell recovery: enrichment × nucleated cell recovery.

did express 7/4 antigen (Table 1), and sorting on the basis of 7/4 antigen expression resulted in the resolution of HPP-CFC from CFC_{CSF-1}. Approximately 24% of HPP-CFC were recovered in the $7/4^-$ fractions compared with <5% of CFC_{CSF-1}, and the ratio of HPP-CFC to CFC_{CSF-1} increased from 0.2:1 in unfractionated marrow to more than 1:1 in the $7/4^-$ fractions.

$7/4^-$ fractions were enriched 10- to 25-fold for HPP-CFC when compared with unfractionated bone marrow (Table 1). The recovery of HPP-CFC in $B220^-$ cell fractions (28.2%) was similar to that obtained in $7/4^-$ cell fractions, but the enrichment of sevenfold HPP-CFC compared with unfractionated bone marrow (Table 2) was somewhat lower than that achieved in $7/4^-$ fractions. Because the 7/4 and B220 determinants define different subsets of marrow cells, these results implied that a cocktail of anti-7/4 and anti-B220 antibodies would simultaneously resolve HPP-CFC from CFC_{CSF-1}, and more effectively enrich HPP-CFC than either antibody alone. In these experiments HPP-CFC had been assayed using CSF-1 plus IL-3 plus conditioned medium from the 5637 human bladder carcinoma cell line as a source of hemopoietin 1 [19]. As a result of the demonstration by Mochizuki et al. [20] that hemopoietin 1 was identical to IL-1_α, and confirmation of this finding in our laboratory [21], all subsequent experiments in this study were carried out using CSF-1 + IL-3 + IL-1_α as the stimulus for HPP-CFC.

Optimal conditions for immunomagnetic bead depletion were established empirically by incubating low-density bone marrow cells with a cocktail of anti-7/4 and anti-B220 antibodies and determining the effect of varying incubation times (Fig. 1) and bead : cell ratios (Fig. 2) on nucleated cell recoveries, and the recovery of HPP-CFC. An incubation

Table 2. Enrichment of normal bone marrow colony-forming cells by discontinuous density gradient centrifugation and cell sorting on the basis of light scatter and B220 surface antigen expression

	Nucleated cell recovery (percent)	Colonies per 2500 cells[a]	
		CFC_{CSF-1}	HPP-CFC
Unfractionated bone marrow	100	78.3 ± 3.4	6.3 ± 0.9
Low-density cells	17.1	107.5 ± 8.0 (23.5%)[b]	17.5 ± 0.3 (47.5%)
Low-density cells selected by light scatter and fluorescence			
$B220^-$	4.1	295.0 ± 25.7 (15.4%)	43.3 ± 6.7 (28.2%)
$B220^+$	1.0	6.7 ± 4.4 (0.1%)	0

[a] Means ± SEM from a single experiment plated in triplicate.
[b] Progenitor cell recovery: enrichment × nucleated cell recovery.

time of 45 min at 4°C with end-over-end rotation at a bead : cell ratio of 10:1 was found to be optimal and has been used routinely for normal bone marrow cell suspensions. Cell densities ranging from 10^6 to 10^7 per ml, or volume of cell suspension did not alter the effectiveness of the separation or the yield of cells obtained (data not shown).

The in vitro colony-forming capacity of the marrow is not affected by incubation with primary antibodies or second-antibody-conjugated immunomagnetic beads alone, or by sham end-over-end rotation (Table 3). Recovery of nucleated cells following incubation with the antibody cocktail averaged 95.7% in the five experiments illustrated in Table 3, and 86.7% ± 3.8% in the series of 25 experiments reported in Table 4. There is some cell loss on incubation with end-over-end rotation, but this is not significantly increased in the presence of anti-rat IgG-coated immunomagnetic beads (Table 3), illustrating that the cell losses observed are nonspecific, and attributable solely to mechanical manipulation of the cell suspension.

Flow cytometric analysis of low-density bone marrow cells incubated with anti-7/4 and anti-B220 antibodies and stained with FITC-conjugated second antibody showed that approximately 85% of nucleated cells are $7/4^+$ and $B220^+$. In comparison, negative immunomagnetic selection of anti-7/4- and anti-B220-labeled cells incubated with goat anti-rat IgG-conjugated Dynabeads defines an average of 78.9% ± 1.6% nucleated cells as $7/4^+$ and $B220^+$. A comparison of the fluorescence intensity of anti-7/4-, anti-B220-labeled cells incubated with FITC-conjugated second antibody before (Fig. 3a) and after (Fig. 3b) immunomagnetic bead depletion showed that the $7/4^+$, $B220^+$ cells remaining after immunomagnetic bead depletion only expressed low levels of these antigens.

The effectiveness of negative immunomagnetic selection in enriching HPP-CFC from normal bone marrow is illustrated in Table 4, which summarizes the results of 25 experiments. A major advantage of immunomagnetic selection is that, in contrast to cell sorting, there is no significant loss of HPP-CFC in the separative procedures after the density

Table 3. The effects of primary antibody, second-antibody-conjugated immunomagnetic bead incubation, and sham end-over-end rotation on progenitor cell recovery

	Nucleated cell recovery (percent)	Colonies per 2500 cells	
		CFC_{CSF-1}	HPP-CFC
Control	100	83.3 ± 22.6	10.5 ± 0.7
Anti-7/4 + anti-B220	95.9 ± 7.1	85.9 ± 17.6[a]	8.0 ± 1.4[a]
Goat-anti-rat coated beads (10–20 beads/cell)	69.6 ± 6.2	90.3 ± 18.2	13.1 ± 2.5
Sham end-over-end rotation	77.6 ± 3.2[a]	81.8 ± 19.7[b]	10.1 ± 3.4[b]

Values are the means ± SEM of five experiments, each plated in triplicate.
[a] Only four replicates could be pooled.
[b] Only three replicates could be pooled.

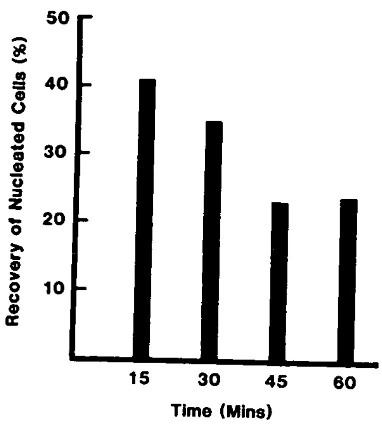

Fig. 1. Determination of optimal incubation time for negative immunomagnetic bead depletion. Aliquots of low-density bone marrow cells were labeled with anti-7/4 and anti-B220 antibodies, and then incubated with anti-rat IgG-conjugated Dynabeads at 4°C end-over-end rotation at a bead:cell ratio of 15:1. Rosetted cells were removed, and cell recovery was determined at various intervals during incubation.

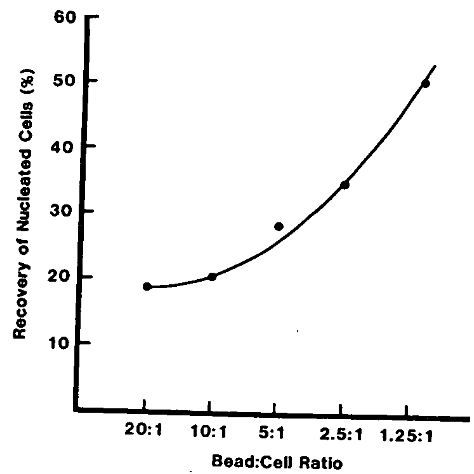

Fig. 2. Determination of the optimal bead:cell ratio for negative immunomagnetic bead depletion. Aliquots of low-density bone marrow cells were labeled with anti-7/4 and anti-B220 antibodies, and then incubated with anti-rat IgG-conjugated Dynabeads for 45 min at 4°C end-over-end rotation at various bead:cell ratios. Rosetted cells were removed and cell recoveries were determined.

gradient centrifugation step ($0.8 < p < 0.9$; t-test). Approximately 65% of HPP-CFC present in the unfractionated bone marrow cell suspension were recovered, in comparison with 22% of CFC_{CSF-1}. The tenfold enrichment of HPP-CFC over unfractionated bone marrow cell levels was comparable to that obtained by cell sorting, and the recoveries of HPP-CFC were threefold higher.

Discussion

Negative immunomagnetic selection using an anti-7/4, anti-B220 antibody cocktail is a rapid and efficient alternative to fluorescence-activated cell sorting for the partial enrichment and purification of primitive hemopoietic progenitor cells from normal bone marrow. The reproducibility and effectiveness of this separation technique is illustrated in Table 4, and is also supported by our experience in >100 separations forming a part of other studies now completed ([21, 22] and Bertoncello et al., in preparation) or in progress using normal marrow, and regenerating marrow from post-5-FU treated mice.

The advantages of negative immunomagnetic selection are numerous. There is no limitation on the number of cells that can be processed in a single separation, whereas enrichment, recovery, and yield of target cells by fluorescence-activated cell sorting is contingent on time and the selection of sort parameters, which inevitably results in a compromise between fold-enrichment and yield. Because of the specificity of the immunomagnetic beads and primary antibodies, enrichment of primitive progenitors is achieved with negligible loss at the bead depletion step (Table 4) and with concurrent resolution of these progenitors from committed progenitor cells of more restricted potential.

The possibility of performing negative immunomagnetic selection without initial separation of marrow cells by discontinuous density gradient centrifugation has been considered, and was rejected for the following reasons. Although this appeared to be an attractive procedure that could counteract the loss of HPP-CFC at the density separation step, erythroid cells would remain as a major contaminant, and the quantity of immunomagnetic beads required to achieve an optimal bead : cell ratio under those conditions would be prohibitive.

Negative immunomagnetic selection can be used to provide a partially purified population of highly enriched primitive progenitor cells from normal or post-5-FU bone marrow [21] that can be stored frozen for the routine assay of hemopoietic growth factor preparations. Because unlimited

Table 4. Enrichment and recovery of $CFC_{CSF\text{-}1}$ and HPP-CFC from normal bone marrow by discontinuous density gradient centrifugation and negative immunomagnetic selection

	Nucleated cell recovery (percent)	Colonies per 2500 cells	
		$CFC_{CSF\text{-}1}$	HPP-CFC
Unfractionated bone marrow	100	35.6 ± 4.9	5.0 ± 0.8
Low-density cells	26.8 ± 1.1	71.7 ± 12.5 (63.1% ± 8.4%)[a]	9.9 ± 2.1 (67.7% ± 12.9%)
Low density + immunomagnetic selection (7/4⁻, B220⁻)	5.7 ± 0.5	103.4 ± 10.6 (22.2% ± 4.7%)	50.0 ± 6.0 (64.8% ± 7.6%)

Means ± SEM from 25 experiments each plated in triplicate.
[a] Progenitor cell recovery: enrichment × nucleated cell recovery.

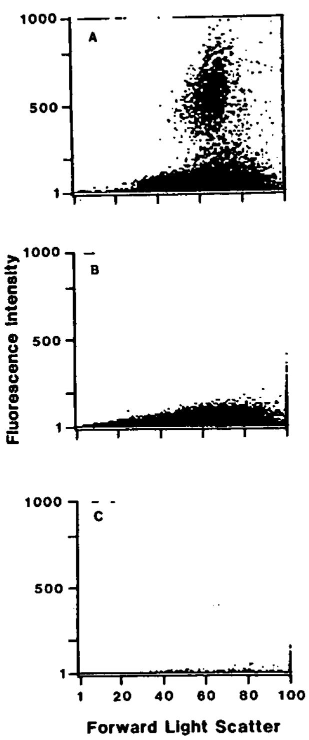

Fig. 3. The effectiveness of immunomagnetic bead depletion is illustrated by comparing the *dot display* of fluorescence versus forward light scatter for low-density bone marrow cells labeled with anti-7/4 and anti-B220 antibodies and FITC-conjugated anti-rat IgG (A) before, and (B) after immunomagnetic bead depletion at 4°C end-over-end rotation at a bead:cell ratio of 10:1. Panel (C) is the *dot display* of bone marrow cells labeled with FITC-conjugated anti-rat IgG alone.

quantities of unfractionated bone marrow can be processed in a single separation with relatively high enrichment and recovery of primitive progenitor cells, negative immunomagnetic selection is a powerful preenrichment step for the further purification of primitive cells by fluorescence-activated cell sorting on the basis of other markers (Bertoncello et al., in preparation). The time required for cell sorting following immunomagnetic selection can be reduced by fivefold or more for the yield of an equivalent quantity of purified progenitors obtained by cell sorting alone, and it is possible to prepare up to 2 × 10^6, or more, highly purified cells for further study in this manner.

The protocol described in this paper should be regarded as a prototype method that will be refined as additional antibodies that can be added to the anti-7/4, anti-B220 cocktail are identified. Recently Kannourakis and Bol [23], and Kannourakis and Johnson [24] have demonstrated that human bone marrow progenitor cells could also be fractionated by immunomagnetic selection.

Acknowledgments

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